In vivo ^{31}P NMR spectroscopic studies of soybean *Bradyrhizobium* symbiosis

Compartmentation and distribution of P metabolites

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In vivo ^{31}P NMR spectroscopy was used to study the distribution of P metabolites and compartmentation in soybean nodules. By careful separation of the cortex, central matrix and bacteroids, we were able to obtain in vivo ^{31}P spectra of the metabolites associated with each specialized section of the nodule tissue. These results indicated that in the earlier stages of growth ($\leqslant$ 4 weeks) the majority of P_i was actively metabolized in the cytoplasm. Furthermore, ^{31}P spectra revealed that the cortical cells contained negligible amounts of phosphorus-containing metabolites in their cytoplasm. However, the vacuoles of the cortical cells appeared to preferentially accumulate P_i during growth. In contrast, the P_i level of the vacuoles of uninfected cells in the central matrix, after 7 weeks, remained relatively constant.

1. INTRODUCTION

Nitrogen fixation in legumes is affected by a range of mineral nutrients [1]. Phosphorus (P) has specific roles in initiation, growth and functioning of nodules, in addition to its involvement in host plant growth processes [2,3]. For example, P deficiency inhibits growth of the nodule more than either the root or shoot [4]. In addition symbiotic dinitrogen fixation has a higher P requirement for optimal functioning than either host plant growth or nitrate assimilation [3]. In these latter studies, the effect of the increased P supply on growth of symbiotic plants was examined indirectly by determining the nitrogenase activity, the biomass or the P concentration in each of the different plant organs, i.e., root, nodule, stem and leaf. However, data concerning the distribution of P metabolites in the different nodule tissues during the growth period were not available.

In our previous study [5] we investigated the methodology for examining the symbiotic state between soybean and *Bradyrhizobium japonicum* using in vivo ^{31}P NMR. Different experimental conditions were used to maintain perfused, respiring, detached nodules in an NMR spectrometer magnet.

The present study of living symbiotic plant tissue was used to assess how P_i was utilized and stored in specific cellular compartments of the microorganisms and plant cells during growth. We showed, for the first time, that in the earlier stage of nodule's growth, P_i was actively metabolized in the cytoplasm of the central matrix. After 7 weeks, P_i was preferentially stored in the vacuoles of the cortical cells whereas the vacuolar P_i of the uninfected cells remained constant.

acidic compartment (fig.1B). Microscopic investigations revealed that only uninfected cells in the matrix contained vacuoles. Therefore, the P_i in the acidic compartment of the central matrix represented vacuolar P_i of uninfected cells. Most of the ^{31}P signal from the resonances representing components within the cytoplasm (nucleotide triphosphates, phosphomonoesters, cytoplasmic P_i) of the whole nodules (A) were still present in the central matrix tissue spectrum (B) including an unidentified compound at 0.37 ppm [5].

The spectrum in fig.1C was representative of 9-week-old split nodules. 87% of the total mobile P_i in these nodules was in the acidic compartment. A ^{31}P spectrum of the carefully excised cortex layer showed that 59% of the total mobile P_i of the nodules resided in the vacuole of the cortical cells (D). Following 9 h of perfusion, the cortical cells were still viable as evidenced by their ability to phosphorylate glycerol (appearance of 3-phosphoglycerol, resonance at 4.82 ppm) added to the perfusion medium (data not shown). In spite of the fact that these cells metabolized substrates such as glycerol, their cytoplasmic volume was too small (cell volume >95% vacuole and 5% cytoplasm) to visualize cytoplasmic nucleotides and phosphomonoesters after 20000 scans.

In vivo ^{31}P NMR was used to determine the relative percentage of mobile P_i in vacuoles of both cortical cells and uninfected cells of the matrix as well as the total mobile P_i in the cytoplasm of the central matrix. In addition, it was possible to estimate the relative quantity of mobile P_i in the bacteroid. Fig.1E shows an in vivo spectrum of isolated bacteroids [6]. The resonance at 2.30 ppm represents the cytoplasmic P_i of the bacteroids corresponding to a pH of 6.85. The bacteroids had a relatively more acidic cytoplasm than the observed pH 7.4 in plant cells. However, these pH differences were not resolved in nodules due to the overlap of the broader peaks. The unidentified peak at 0.37 ppm is clearly evident in the bacteroid spectrum. Isolated peribacteroid units (PBU) [10,11], exhibited spectra similar to the bacteroids with no evidence of an acidic compartment containing P_i (data not shown). The peribacteroid space, which surrounded the bacteroids within the peribacteroid membrane, did not appear to contain measurable amounts of mobile P_i.

After demonstrating that spectra of the various compartments could be resolved by the above methodology, the distribution of P_i and P metabolites in the nodules during growth was studied. The relative distribution of cytoplasmic and vacuolar P_i in nodules was compared in 4–12-week-old nodules (table 1). In the earlier growth stages (4 weeks old), most P_i was found in the cytoplasm (61%). After 7 weeks, the majority of P_i (77%) was stored in the vacuole. Since most P_i contained in the nodules at 4 weeks was present in the metabolically active cytoplasm of the central matrix, absorption of P_i probably did not greatly exceed the cell's needs at this time. However, after 7 weeks the proportion of P_i in the vacuole, the site of lower metabolic activity, steadily increased. The increase of P_i in this pool of lower metabolism probably indicated the supply of P_i was exceeding the cell's needs. Throughout the growth period from 4 to 12 weeks the vacuolar P_i of the uninfected cells remained relatively constant at 32% of total P_i. However, P_i continued to be accumulated in the vacuole of the cortical cells (61% of total P_i for the 12-week-old nodules).

We have demonstrated that in vivo ^{31}P NMR spectroscopy can be used to assess the distribution of P_i and P metabolites in living symbiotic soybean root nodules throughout their period of growth. However, it was not possible to determine, in nodule spectra the distribution of P_i in the infected cells. Following their isolation, the bacteroids exhibited a P_i representing a more acidic cytoplasm than host cells (due possibly from damage to the cells upon exposure to air). However, since this P_i resonance was not resolvable in the nodule spectra direct quantification of the bacteroid P_i resonance in spectra of this intact matrix was not attainable. Presently, it is not feasible to determine an exact correlation between the number of bacteroids in

Table 1

Distribution of mobile P_i[a] in *Bradyrhizobium* soybean nodules

	Weeks after inoculation				
	4	7	8	9	12
Total cytoplasmic P_i	61	23	12	13	7
Total vacuolar P_i	39	77	88	87	93
Vacuolar P_i in the cortical cells	21	43	57	59	61
Vacuolar P_i in the uninfected cells (in matrix)	18	35	31	28	32

[a] In % of total P_i, values are approx. ±5%